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VENATION AND SENESCENCE OF POLYEMBRYONIC CITRUS PLANTS

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The evidence for senescence in plants has until recently been *nil.* Benedict (1915) has, however, brought forth a considerable amount of data indicating that the venation in leaves of woody plants is greatly influenced by the age of the plant. As the plant passes from youth to old age, the time being reckoned since its origin from seed, the areas of leaf tissue enclosed by the smallest branches of the fibrovascular bundles become relatively smaller. These he terms vein-islets. In other words, the proportion of fibrovascular tissue in the leaves increases with the age of the plant. Propagation by cuttings, furthermore, does not alter at all the venation in the leaf. It is only when reproduction is by seed that a juvenile venation is obtained.

In view of this fact it seems desirable to ascertain whether or not a seedling produced apogamously shows any signs of senescence as compared with a seedling produced from a fertilized egg. Fortunately we have in various species of Citrus a phenomenon which makes these plants desirable for such an investigation. A single seed may produce more than one seedling—a condition known as polyembryony. One of the embryos is produced as a result of sexual fusion, the additional embryo or embryos by budding of the nucellus. Therefore if any differences are found in the seedlings or plants produced from a single seed, some evidence would be available indicative of the significance of sexual and apogamous reproduction in rejuvenation processes.

POLYEMBRYONY

The term polyembryony means, of course, the presence of many embryos in a seed. This condition is not altogether unusual, yet the great majority of seeds produce upon germination but a single plant, *i.e.*, they are not polyembryonic.

Strasburger (1878), who made an extensive and detailed study of this phenomenon from the cytological and morphological standpoint, names the following plants which exhibit polyembryony: *Santalum album*, *Sinningia*

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lindleyana, *Nothoscordum fragrans*, *Citrus* species, *Mangifera indica*, *Evonymus latifolius*, *Coelebogyne ilicifolia*, *Epheara* sp., *Cheiranthus cheiri*, *Vicium album*, and certain orchids. Coulter, Barnes, and Cowles (1910) state that polyembryony is much more common among gymnosperms than among angiosperms.

The most important question connected with polyembryony has to do with the origin of those plants produced in excess of the normal number from a single seed. When seeds normally produce but a single embryo, this is usually considered to arise from the fertilized egg within the embryo sac, being, therefore, gametic. In Citrus species, according to Strasburger (1878), one plant arises in this manner, while the others arise from the nucellar tissue. The latter, therefore, are apogamous. In other species, variations from this type of polyembryony are described. For example, in *Santalum album* and in *Cypripedium Caleolis* there are two fertilized eggs which develop. But in Citrus species, no other origin than that already noted has ever been found. This, however, does not mean that, when Citrus seeds produce only one plant, that one is always of gametic origin. In fact, Strasburger makes it clear that there is no way of distinguishing the gametic from the apogamous seedlings at the time of their germination except by sectioning. Sometimes one and sometimes the other emerges first, and in other cases only the apogamous plant develops. Osawa (1912) confirms these observations.

In some cases a marked difference in the size of the polyembryonic seedlings is noted. This question s deferred for discussion later.

Methods and Materials

Plants. The seeds, from which the polyembryonic seedlings were grown for this study, were taken from grape fruit secured at the local market. These seeds were planted, one in each pot, and allowed to grow in the greenhouse. The first lot were planted by Dr. Knudson and were two years old at the time this study was undertaken. All of the determinations on the venation character were made from leaves of this lot. A second lot was planted later from which other determinations on relative size of plants, etc., were made.

That similar environmental conditions surrounded the polyembryonic seedlings which were to be compared is quite well insured, since both the plants from a given seed germinate at the same time and develop under identical conditions so far as factors which may be controlled are concerned. One factor which undoubtedly plays an important part in determining the relative size of the polyembryonic plants is not controllable. It has to do with the size of the cotyledons with which each plant from a single seed is provided. This will be considered later.

Clearing and Staining. In order to study the venation of Citrus leaves, it was necessary to devise a method of clearing and staining so that the minutest bundle endings might be readily seen under the microscope.

An examination of the literature revealed only one or two methods for clearing leaves. DeVries (1878), working with potato leaves, gives the following directions for clearing: "Decolorize them in alcohol, treat for a longer time with caustic alkali, and finally wash with water and a dilute solution of acetic acid. The leaf is then put in glycerine and upon standing some time becomes transparent." The method described by Stevens (1907) involves, likewise, the use of alcohol for extracting the chlorophyll. This is wholly inadequate for the thick, leathery leaves of the Citrus species. Therefore a new method was perfected which, with a little modification, was found to be practical in clearing all kinds of leaves.

Entire leaves, if small, or portions of large leaves were placed in a basin of water and boiled for some time. This treatment is necessary in order to remove water-soluble materials, such as tannin, which prevent an even distribution of the stain.

After the leaves had taken on a water-soaked appearance, they were transferred to a side-tube suction flask. This contained 100 cc. of 85 percent nitric acid.[1] The flask was tightly stoppered, and, by means of a Richard's air pump, a partial vacuum was maintained. Such a process accelerated the penetration of the acid and at the same time drew off the escaping fumes which, if allowed to remain, caused a blistering of the tissue.

The leaves were allowed to remain in the acid in vacuum from twenty-four to thirty-six hours or until they had become transparent. They were then washed in running water for six hours, dehydrated, and introduced into the stain. After trying a number of stains, *e.g.*, fuchsin, orange G, safranin, iodine green, neutral red, and methylene blue, the last was found to be by far the most satisfactory. An alcoholic 1 percent solution was round to stain the lignified tissue a deep blue, leaving the remaining parts relatively free from stain. The usual time allowed for staining was twenty-four hours. Decolorizing in acid alcohol was often necessary.

Then, following complete dehydration in absolute alcohol and subsequent clearing in xylol, the leaves were mounted in castor oil. This medium has the following advantages over balsam or glycerine for this work: (1) it penetrates more readily than balsam and is easier to handle, and (2) it will not take up water as will glycerine. These mounts were made only for temporary usage, yet they are still in good condition at the end of two year's time. If permanent mounts are desired, balsam may be used to seal the edges.

Modification of the Method for Thin Leaves. The method described above was employed in treating thick, leathery leaves. For thin, succulent ones, like grape leaves, the following modifications were necessary to insure success:

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[1] Benedict (unpublished) used concentrated nitric acid for clearing. After allowing the leaves to remain in the acid for a while they were removed to a slide and floated in glycerine. The slide was then heated over a Bunsen flame until the leaves were transparent. This method is objectionable because the leaves usually go to pieces.

1. The nitric acid must be diluted to about 50 percent.
2. The length of time necessary for clearing is shorter.
3. The stain should be diluted to 0.5 percent.
4. Complete penetration of the stain is effected in about 12 hours.

In other respects the procedure is the same as that given for the heavier leaves.

Examination of Material and Determinations. All the determinations of vein-islets were made by projecting the magnified image of the stained vascular system by the use of a micro-projection apparatus. This consists of a horizontally placed microscope through which a stream of light, derived from an electric arc, is permitted to pass. The section to be studied was placed upon the stage of the microscope. The light is absorbed by the stained bundles and they appear as a shadow upon white paper. The paper was always placed at the same distance from the ocular of the microscope in making determinations. Adjustment was made so that a piece of paper 75 mm. square would just take a projected image from 4 sq. mm. of leaf surface. Now by marking with a lead pencil in the center of each of the vein-islets, the number of islets in 4 sq. mm. of leaf surface was quickly and accurately determined. The average area of each islet, then, was computed by dividing 4 sq. mm. by the number of vein-islets in that area.

PERCENTAGE OF SEEDS PRODUCING POLYEMBRYONIC SEEDLINGS

Webber (1900) states that the majority of Citrus seeds produce polyembryonic seedlings. Aside from this statement no data are available on this point. Therefore some germination tests were conducted as follows tin basins were filled nearly full of sawdust which had been previously washed and sterilized. Upon this a layer of cheesecloth was laid and the seeds were placed upon the cheesecloth. Then another layer of cloth and more sawdust were added. The seeds prior to planting were treated for six hours in a 7 percent solution of calcium hypochlorite. These precautions eliminated a good many of the contaminating fungi.

It was found that seeds subject to desiccation in storage for only ten days had lost their vitality. Therefore all the seeds for this test were taken from the grapefruit and planted immediately. Germination began after a period of five days and continued for fourteen days.

Table 1 is a summary of these tests.

The results show that a high percentage of the fresh seeds are viable. Despite the precautions taken against contamination, the majority of the seeds which did not germinate were infected. This was indicated by the fact that these seeds rotted, and also by the fact that a higher percentage of germination was obtained when the seeds germinated in pure culture.

TABLE 1. *Percentage of germinating seeds of Citrus grandis producing polyembryonic seedlings*

Total No. Seeds Used in Test	Percentage of Seeds that Germinated Producing				Total Per-cent Germinated	Total Percent Producing Polyembryonic Plants
	1 Hypocotyl	2 Hypocotyls	3 Hypocotyls	4 Hypocotyls		
888	48.82	37.8	5.9	4.2	92	43.18

The interesting facts to be noted, however, are that nearly half of the seeds which germinated produced more than one plant each, and that only four seeds out of the entire lot produced four seedlings each and the great majority produced but two each.

INFLUENCE OF CERTAIN FACTORS ON VENATION

Schuster (1908) has contributed more of real value as regards the effects of environmental conditions upon venation than any other writer upon this subject. Benedict (1915) has verified some of these findings in respect to *Vitis vulpina*. Zalenski (1902) did not confine his comparisons to a single species, so that hereditary variations were not eliminated. All the work, however, published by these writers shows that there are certain variations attributable to environmental causes. These should, then, so far as possible, be eliminated before a comparison of the venation of polyembryonic seedlings is undertaken.

UNIFORMITY IN SIZE OF VEIN-ISLETS IN DIFFERENT PARTS OF A LEAF

Schuster (1908) points out that there is a marked uniformity in the size of the vein-islets in all parts of the leaf except near the midrib and at the apices. The shape of the vein-islets in leaves of the grapefruit varies greatly in different regions of the leaf. Near the midrib they are long, narrow, and rectangular, and gradually become more nearly circular toward the periphery. Here a coalescence of the secondary bundles occurs, and the vein-islets are quite circular. Because of this difference in shape it was thought advisable to make determinations as to the size of the islets in the regions of the periphery and of the midrib respectively. The results of these findings are summarized in table 2. Sixty leaves were examined and four determinations were made from each region in each leaf. Hence, the mean averages represent a total of 240 determinations. In every table following, the figures representing the size of the vein-islets of a single leaf are the averages of four determinations.

The data show that the size of the vein-islets is independent of their shape or place in the leaf. Therefore, in later work, determinations were taken from either the periphery or the region of the mid-rib.

TABLE 2. *Showing uniformity of vein-islet area in different regions of the same leaf (Citrus grandis)*

Leaf No.	Number of Vein-islets in 4 Sq. Mm. of Leaf Surface		Size of Vein-islets (Sq. Mm.)	
	Midrib	Periphery	Midrib	Periphery
1	12	12.5	.3333	.3250
2	13	13	.3123	.3123
3	13	11	.3123	.3666
4	10	11	.4000	.3666
5	12	13	.3333	.3123
6	14	16	.2894	.2500
7	12	13	.3333	.3123
8	13	13	.3123	.3123
9	8	8	.5000	.5000
10	11	11	.3666	.3666
Average	11.8	12.1	.3492	.3423
Average 50 additional leaves	10.5	10.6	.3809	.3775
Mean average 60 leaves	10.7	10.9	.3756	.3714

INFLUENCE OF AN INSUFFICIENT FOOD SUPPLY UPON THE SIZE OF VEIN-ISLETS

To some of the pots in which the Citrus seedlings were grown, no fertilizer was applied. The soil was sandy and very poor in humus content. These plants were noticeably below the normal size of the other two-year-old seedlings. They were also chlorotic, and on the whole gave a very sickly, stunted appearance. During the course of the summer, some of these plants sent their roots through the drain hole in the pot and established themselves in the soil upon which the pots were placed. These plants at once responded to the effects of an adequate food supply and grew rapidly. The new leaves had a perfectly normal color and size, and the old leaves regained in part a normal color.

Mature leaves were taken from these stunted plants and the venation was studied. The results are given in table 3, where the size of the vein-

TABLE 3. *Influence of insufficient food supply upon size of vein-islets of leaves of Citrus grandis*

Plant Number (2 Leaves per Plant)	Number of Vein-islets in 4 Sq. Mm.		Size of Vein-islets (Sq. Mm.)	
	Chlorotic	Healthy	Chlorotic	Healthy
1	12	10	.3333	.4000
2	13	10.5	.3123	.3800
3	13	10.2	.3123	.3956
4	10	11.5	.4000	.3570
5	12	11.5	.3333	.3333
6	14	12	.2989	.3333
7	12	12	.3333	.3752
8.–	8	10.8	.5000	.3666
9	11	11	.3666	.3800
10	10	10.5	.4000	.3405
Average for 10 plants	11.5	11.00	.3590	.3661

islets of leaves from normal plants is compared with the same character in leaves from the chlorotic ones.

While the vein-islets in healthy leaves showed an average size slightly smaller than the size of those in the chlorotic leaves, the difference is well within the range of variations noted in individual leaves in each series. Hence the conclusion is warranted that vigor and nutrition have no measurable effect upon the size of the vein-islets (figs. 1 and 2).

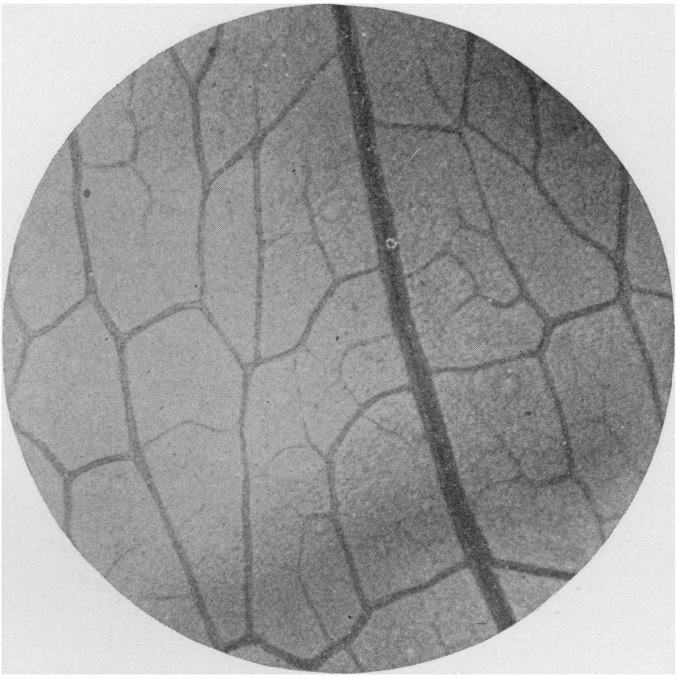

FIG. 1. Photomicrograph showing the venation in a mature chlorotic leaf of *Citrus grandis* measuring 16 mm. in short diameter. × 30.

INFLUENCE OF THE SIZE OF LEAVES UPON THE SIZE OF VEIN-ISLETS IN CITRUS GRANDIS

One of the most common variations with which one meets is in the size of mature leaves borne by a single plant. The polyembryonic Citrus seedlings are no exception. Experiments were accordingly made to ascertain whether this variation in size of the leaf was in any way correlated with the size of the vein-islets.

Only mature leaves were used, but the size of the leaves varied from 10 millimeters up to 68 millimeters in width. Only the short diameter of each leaf is given, taken at right angles to the midrib at the point of maximum width. The long diameter, *i.e.*, parallel with the mid-rib, was usually two times the short diameter. The results are given in table 4. The table shows that the size of the vein-islets in these leaves is quite constant irrespective of the great differences in the size of the leaves.

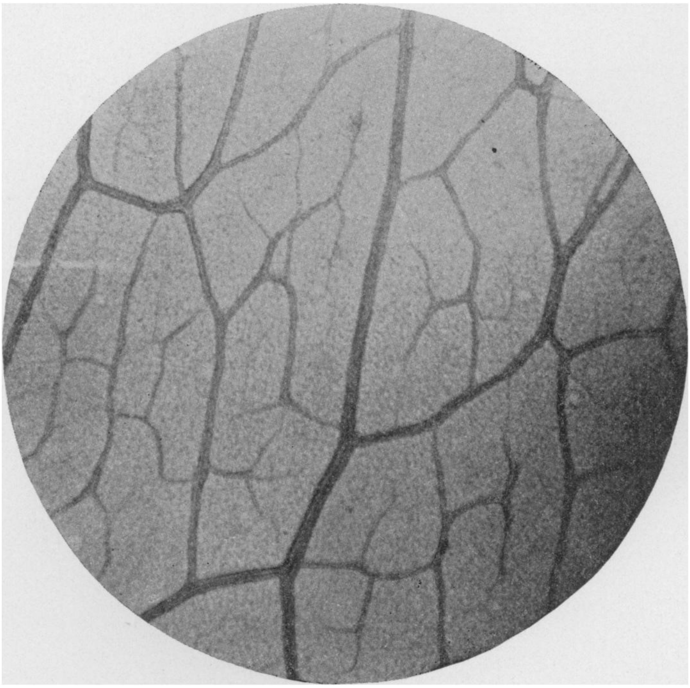

FIG. 2. Photomicrograph showing the venation in a mature healthy leaf of *C. grandis* measuring 68 mm. in diameter. × 30.

TABLE 4. *Influence of size of leaves upon size of vein-islets in leaves of Citrus grandis*

Leaf No.	Size of Leaf (Mm.), Short Diameter		No. of Vein-islets in 4 Sq. Mm. of Leaf Surface		Size of Vein-islets in Mm.	
	Small	Large	Small	Large	Small	Large
1	20	40	11.5	10.7	.3405	.3740
2	25	38	10	10	.4000	.4000
3	12	40	9.2	11	.4460	.3666
4	22	42	11.2	13.2	.3530	.3040
5	27	33	9.2	11.7	.4460	.3400
6	13	35	11.2	14	.3530	.2894
7	16	40	12.7	9.2	.3199	.4460
8	20	40	11.5	9.7	.3499	.4267
9	18	47	11.7	10.7	.3405	.3740
10	15	40	10.5	10.7	.3800	.3740
Average	18.8	39.5	10.87	11.09	.3728	.3694
Mean average for 25 leaves	17	45	10.7	11	.3740	.3666

INFLUENCE OF THE MATURITY OF THE LEAVES UPON THE SIZE OF THE VEIN-ISLETS IN CITRUS GRANDIS

There seem to be no data published concerning the duration of time that the leaves persist upon grapefruit plants. Some of the plants at the greenhouse are now three years of age and no appreciable leaf drop has been noted. A leak of illuminating gas defoliated some of the plants of other species in this house, but the injury, if any, to the grapefruit was not sufficient to be noticeable. New growth is terminal and begins in the greenhouse early in March following a rest period.

In some of the preliminary work, which was done during the summer of 1916, it was noted that among five or more leaves taken from a single plant, one or sometimes two would occasionally show vein-islets very much smaller than those in the other leaves. In order to determine the meaning of this discrepancy the following method was employed. Beginning with the topmost leaf and proceeding downward to the last leaf at the base, a series of leaves was taken which represented the oldest and youngest leaves upon the plant and a gradual gradient from one to the other. In most cases sixteen leaves comprised such a series, and their individual venation was determined. Table 5 gives the data from one series. The others were all comparable with this one.

TABLE 5. *Variation in size of vein-islets in leaves of Citrus grandis owing to different degrees of maturity*

Leaf No.	Diameter of Leaves Mm.		No. Vein-islets per Unit Area (4 Sq. Mm.)	Size of Vein-islets (Sq. Mm.)
	Short	Long		
1	8	23	103	.0388
2	10	28	98	.0408
3	11	30	72	.0554
4	18	36	67	.0597
5	24	50	54	.0741
6	32	60	41	.0975
7	39	80	27	.1483
8	30	70	20	.2000
9	48	110	13	.3123
10	45	82	12	.3333
11	20	35	11.5	.3499
12	40	70	10.8	.3860
13	30	55	9	.4500
14	35	50	9.5	.4730
15	30	25	10	.4000
16	20	40	12	.3333

Here for the first time a positive correlation is evident (figs. 3 and 4). The most immature leaf in which a differentiation of the bundles has occurred[2] contains the greatest number of vein-islets per unit area. Furthermore, it appears that with increasing maturity there is a corresponding increase in the size of the vein-islets until maturity is reached.

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[2] Nägeli (1855) and Prantl (1883) have shown that the nervature of certain dicotyledonous leaves is not completely performed in the bud, but that new veins arise as the leaf expands. This is the case in leaves of *Citrus grandis.* The smallest leaves in which the bundles would take a differential stain were those measuring not less than 8 millimeters (short diameter).

Twenty immature leaves of varying size showed a very marked variability in the size of the islets, while in the mature leaves they tended to be constant. The immature leaves are distinguishable from the mature also by their lighter green color, and by a more resinous feel.

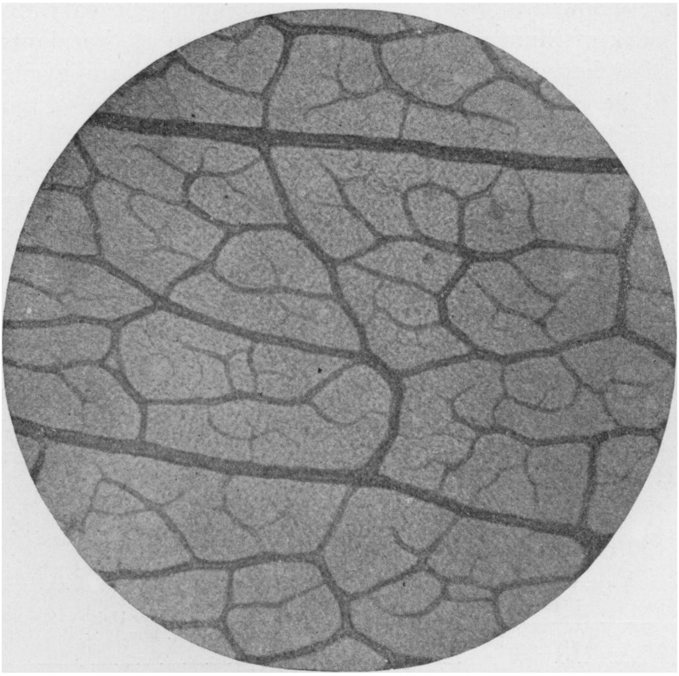

FIG. 3. Photomicrograph showing the venation in an immature leaf of *Citrus grandis*. × 30.

One may conclude, therefore, that the main skeleton work of the vascular system in the leaf is laid down quite early; that little additional differentiation takes place after this as the leaf continues to expand, and that the spaces between the bundles consequently increase in size until the leaf is mature. The bundles themselves become much larger in cross section as the leaf advances toward maturity. In very young leaves the stained bundles appear as fine blue threads in striking contrast to the large, rigid veins found in mature leaves. One thing which seems inexplicable is the fact that the plant seems to predetermine how much differentiation shall be allowed this or that leaf, so that at maturity the number of intersecting veins in a unit area of leaf surface shall be more or less nearly constant, independent of the size of the leaf.

VARIATION IN THE SIZE OF VEIN-ISLETS IN POLYEMBRYONIC SEEDLINGS OF CITRUS GRANDIS

Since some of the more variable factors have been eliminated, the way seems clear to take up the principal question: Is there any difference between the polyembryonic seedlings as regards the character of venation?

Only mature leaves were used. The larger plant was labeled *A* and the smaller one *B*, when two were produced from a single seed. When the plants were equally large and vigorous, the lettering was purely arbitrary.

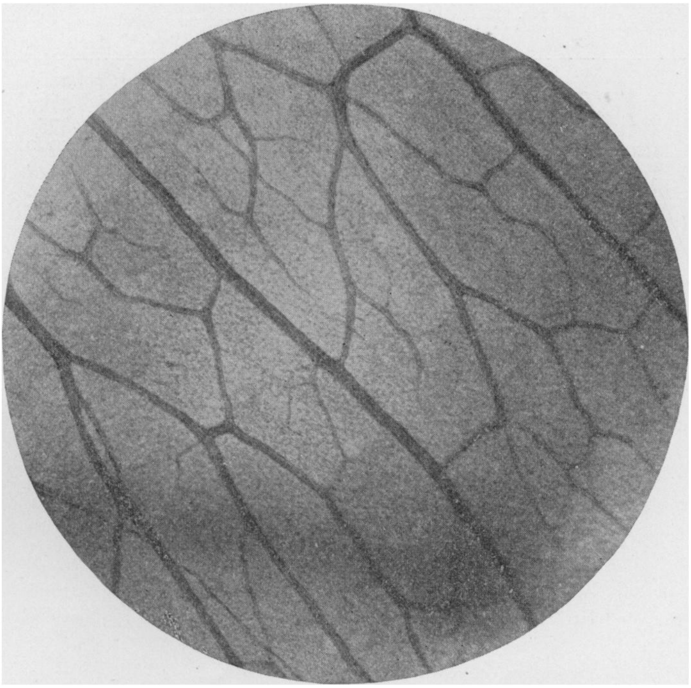

FIG. 4. Photomicrograph showing the venation in a mature leaf of *Citrus grandis*. × 30.

Altogether 100 leaves were examined from each of the *A* and *B* series. This represents 20 plants and 5 leaves from each plant.

The only conclusion which these data warrant is that, so far as the venation test for senility is concerned, it has failed to reveal any essential differences between polyembronic seedlings of *Citrus grandis*. While there is no way of knowing positively the number of polyembryonic plant pairs used in this study composed of individuals having apogamous and gametic origin respectively, yet there can be little doubt but that the leaves from such pairs were encountered and studied in one phase or another of the work. Since no marked differences in vein-islet area were discovered, the following possible explanations are offered: (1) Since the age of the parent plant yielding the seed is not known, it is possible that the difference between parent and seedling is not great enough to be measurable by this test. (2) The venation character may not be an indication of age differences in this species.

TABLE 6. *Size of vein-islets in leaves from polyembryonic seedlings of Citrus grandis*

Leaf No.	Number of Vein-islets in 4 Sq. Mm.		Size of Vein-islets (Sq. Mm.)	
	Plant *A*	Plant *B*	Plant *A*	Plant *B*
1	10	9.2	.4000	.4431
2	10.5	10.2	.3800	.3920
3	10.8	11	.3750	.3666
4	10	11	.4000	.3666
5	11.5	8.8	.3499	.4605
Ave. 1st pair plants	10.56	10.04	.3809	.4057
1	13.7	10.5	.3040	.3820
2	14	14	.2894	.2894
3	13.5	12	.2955	.3333
4	11.7	13.7	.3500	.3050
5	11.5	11	.3499	.3666
Ave. 2d pair plants	12.88	12.24	.3177	.3352
1	13	13	.3123	.3123
2	13.7	15.8	.2999	.2558
3	10	14	.4000	.2894
4	11.5	13	.3499	.4000
5	——	10	——	.4000
Ave. 3d pair plants	12.05	13.16	.3405	.3315
1	10	10.2	.4000	.3820
2	8.8	10.7	.4605	.3750
3	10.2	9.8	.3920	.4160
4	11	10	.3666	.4000
5	10	11	.4000	.3666
Ave. 4th pair plants	10.2	10.34	.4038	.3879
1	10.2	10	.3920	.4000
2	9.8	10	.4270	.4000
3	11	11.8	.3666	.3400
4	11	11	.3666	.3666
5	10.8	10.2	.3741	.3920
Ave. 5th pair plants	10.56	10.60	.3852	.3797
Mean ave. for 25 leaves—5 pairs of plants	11.29	11.15	.3655	.3680
Mean ave. for 100 leaves—25 pairs of plants	11.1	11.0	.3624	.3666

The reasons that may be advanced in favor of these suggestions are: 1. The grapefruit was not grown in Florida on an extensive commercial scale prior to the big freeze of 1894.[3] At most, then, the bearing trees are probably not more than twenty-five years old. Hence the gametic seedlings, which are assumed to be rejuvenated, may not be far enough removed in age from the apogamous seedlings, which are senile to the same degree as the parent, to show a measurable difference in the size of vein-islets.

[3] Bailey, L. H., Encyc. Hort. 2: 782.

Opposed to this is the fact that Benedict (1915) reports an evident difference in cases in which the difference in age was only approximately three years. A great deal of his data are derived from plants showing an age difference of no more than ten to twenty years.

2. It is possible that in *Citrus grandis* the venation character is not correlated with age. Yet Benedict found such a correlation in twelve species of woody plants, and he states that he believes it to be of quite universal application.

A third possible explanation is given in the discussion of results.

NUCLEO-CYTOPLASMIC RATIO

Inasmuch as the polyembryonic seedlings showed no differences from a morphological standpoint, it seemed advisable to try one other test for senescence—the nucleo-cytoplasmic ratio.

To Hertwig (1903) and Minot (1908) we are indebted for the hypothesis that there is a definite correlation between the relative volume of nucleus and the volume of cytoplasm in cells as an organism passes from youth to old age. Minot holds that the volume of the nucleus is much larger in proportion to the volume of cytoplasm in cells of a young organism, and that, as age advances, the volume of cytoplasm gradually increases with a corresponding decrease in the volume of the nucleus.

Hertwig held a view diametrically opposed to that of Minot.

Conklin (1912) contends that such a ratio does not hold even in cells of the same tissue, for the ratio varies with the different phases of mitosis.

The meristematic tissue of the root tips of polyembryonic seedlings was used. The seeds were germinated in pure culture, in test tubes on agar. They were first sterilized by removing the outer husk and allowing the naked seeds to stand in 7 percent hypochlorite of lime for five hours.

The hypocotyls were allowed to grow until they were from ten to fifteen millimeters long. At this time the polyembryonic seedlings from single seeds showed differences in length of the hypocotyls in many cases. Those seedlings which showed this difference were the only ones used in this study, and as in the previous test the larger one was termed *A* and the other *B*.

Treatment. The root tips were killed in 1 percent chrom-acetic acid solution. They were allowed to remain in this solution for twenty-four hours, at the end of which time they were washed in running water for an equal period. The subsequent treatment for fixing and clearing is that recommended by Chamberlain (1915) for root tips. The sections were cut longitudinally 10 μ in thickness and stained with Heidenhain's iron-alum haematoxylin.

All determinations of the nuclear and cell diameters were made under the oil immersion objective. Inasmuch as a very rapid vacuolization of the cytoplasm occurs as the cells become more and more differentiated, no better method of insuring comparable volumes could be hit upon than by taking those cells in the cortex at the same distance from the base of the root cap and which contained nuclei that were round in section. The volume of the cytoplasm was assumed to be that of the cell minus the volume of the nucleus. The volumes were computed on the basis that the cells were cylinders, and the nuclei spheres.

Fourteen root tips from each of the *A* and *B* hypocotyls were examined. In table 7, these data are summarized. The figures representing the volume of nucleus and cytoplasm, respectively, for each root tip, are averages of fifteen to twenty-five determinations.

TABLE 7. *Summary of determinations of nucleo-cytoplasmic ratios made from each pair of polyembryonic root tips of Citrus grandis*

No. of Root Tip.	Volume Nuclei (Cu. Microns)		Volume Cytoplasm (Cu. Microns)		Nucleo-cytoplasmic Ratio	
	A	B	A	B	A	B
1	166.89	187.05	626.06	544.13	1 : 3.9	1 : 2.9
2	195.02	187.05	881.04	544.13	1 : 4.4	1 : 2.9
3	166.89	164.52	1,168.47	826.81	1 : 7	1 : 5
4	187.05	187.05	1,397.27	1,265.36	1 : 7	1 : 6.7
5	166.89	142.03	1,168.47	868.23	1 : 7	1 : 6.1
6	130.58	187.05	1,189.14	1,113.80	1 : 9	1 : 5.9
7	187.05	164.52	778.67	566.66	1 : 4	1 : 3.4
8	142.03	194.99	687.64	994.93	1 : 4.8	1 : 5
9	160.69	180.75	1,123.38	658.11	1 : 7	1 : 3.9
10	226.18	191.94	1,421.63	672.22	1 : 6	1 : 3.9
11	180.75	154.20	1,428.39	843.04	1 : 7.9	1 : 6
12	194.99	140.26	686.72	1,430.70	1 : 3.5	1 : 10
13	166.97	157.08	1,053.67	1,414.04	1 : 6.3	1 : 8
14	174.59	173.19	1,396.88	1,267.60	1 : 8	1 : 7
Mean . . .	174.75	171.52	1,069.60	859.30	1 : 6.3	1 : 5.0

The results show that the nucleo-cytoplasmic ratio as determined by this method is subject to considerable variation. This may be explained in part by the fact that the percentage of vacuolization of the cytoplasm at an equal distance from the apical cell of the meristem is not the same in every root tip. It is, obviously, impossible to make the necessary corrections, for there is no way of knowing the degree of differentiation that has taken place. The only possible way to overcome this difficulty is to take mean averages of many determinations. The mean averages for fourteen root-tips examined show that in a measure this error has been overcome. It also shows that the nucleo-cytoplasmic ratio is not appreciably different in the *A* and *B* root-tips. Certainly the difference is not as great as the individual variations in either case.

SOME PHYSIOLOGICAL CONSIDERATIONS

Since the two tests which have gone before have revealed no apparent difference between the seedlings arising from a single seed, the question then naturally arises: Do the polyembryonic seedlings from single seeds show any differences in size and vigor? If there is a difference, to what is it due?

Webber (1900) found that, when *Citrus aurantium* was crossed with *C. trifoliata*, among polyembryonic seedlings one plant would usually show intermediate characters common to both parents. This seems to leave no doubt but that such a plant had its origin from the fertilized egg. This hybrid, furthermore, was usually larger than the other plants according to Webber, who attributes the difference in vigor to the beneficial results of crossing. This is explicable on the ground that when hybridization is effected between parents of distinctive characters, new combinations of characters often result which are more favorably adapted to the environment than are those of either parent. This greater vigor has often been mistaken for an indication of rejuvenescence.

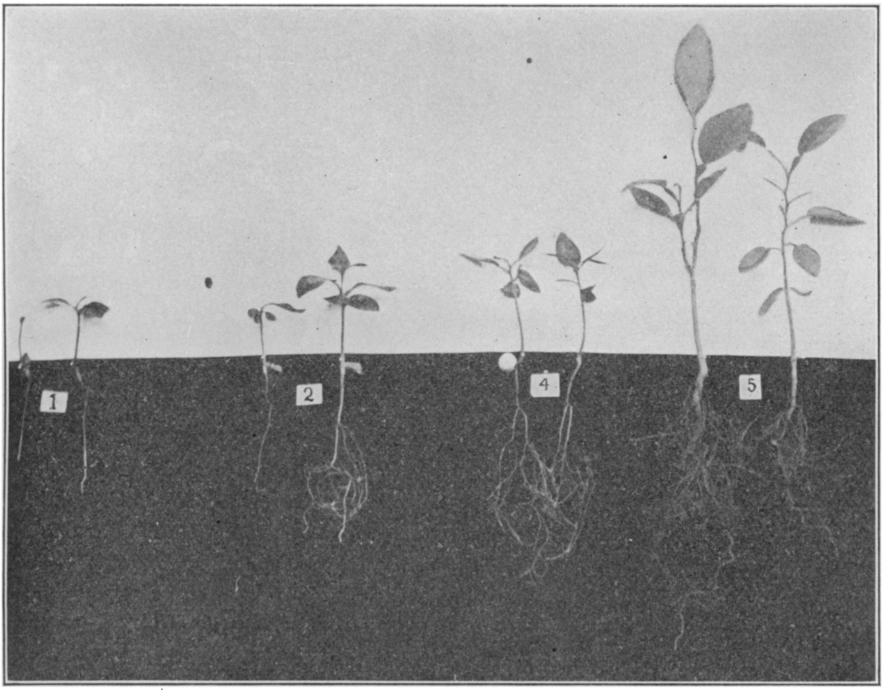

FIG. 5. Photograph showing 4 pairs of polyembryonic seedlings of *C. grandis*. Nos. 1 and 2 show polyembryonic plants of unequal size. The relative size of attendant cotyledons in no. 2 are easily seen. The size of cotyledons is evidently correlated with the size of the plants. No. 4. Polyembryonic plants of equal size and vigor (one-year-old seedlings). No. 5. Polyembryonic plants of equal size. They are three years old and chlorotic.

Among all the seedlings of *Citrus grandis* grown in the greenhouse, there were no true hybrids, as far as the writer observed. Yet in many cases two plants from a single seed showed differences in size. In other cases both plants developed equally well. Of 36 pots containing two seedlings each, 40 percent contained seedlings of substantially equal size and apparent vigor. The remaining 60 percent showed plants of unequal size in all gradations.

At the time the germination tests were made, it was a matter of common observation that each polyembryonic seedling had its pair of cotyledons attached. In case three or four embryos developed from a single seed, some of the last to germinate had cotyledons not much larger than a pin head. On the other hand, some of the others were subtended by cotyledons that comprised the major portion of the entire seed. Although no specific data were taken on this point, it seems only reasonable to conclude that the size of the germinating seedling is directly correlated with the size of the cotyledons, or the amount of food. The general observations substantiate this view (figs. 5 and 6).

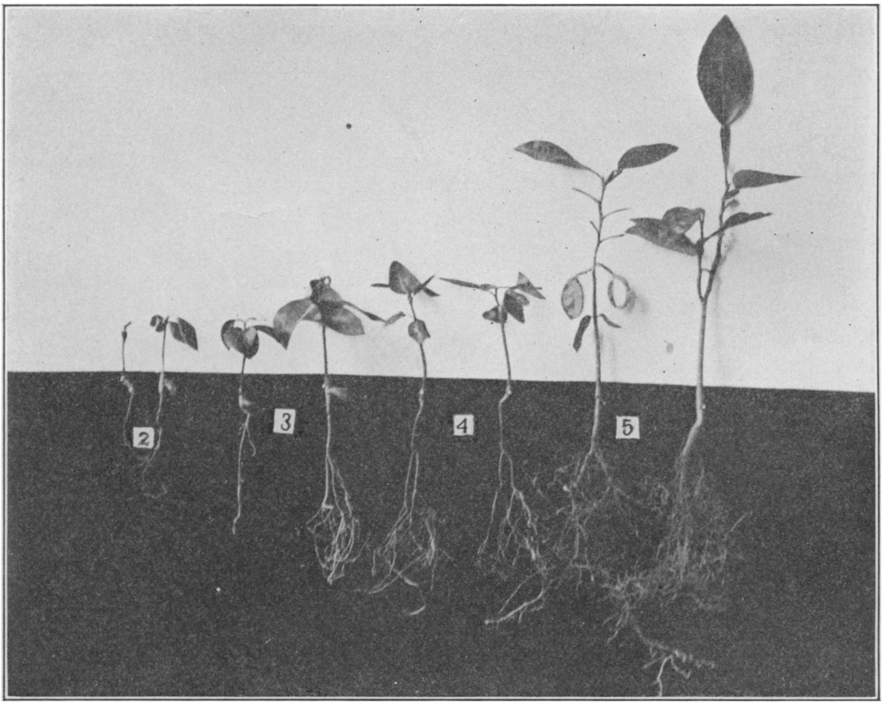

FIG. 6. 4 pairs of polyembryonic seedlings of varying age and size. No. 2. Polyembryonic seedlings 5 months old, of unequal size and vigor. No. 4. Polyembryonic seedlings 11 months old, of equal size and vigor. No. 5. Polyembryonic seedlings 3 years old, of about equal size and vigor.

Strasburger (1878) showed that sometimes the apogamous embryo develops first and much more rapidly than the one arising from the fertilized egg; that in some cases the former, wholly or partially, suppresses the development of the latter. Osawa (1912) states that it is very common for as many as nine embryos to be present in the embryo sac, yet only rarely do more than four develop.

These observations all tend to show that the relative size and vigor of polyembryonic plants is primarily a matter of priority of development and food content of the cotyledons. The embryo, whether gametic or apogamous, which has the initial start and the greater reserve of food will probably produce the larger plant. In those cases in which the embryos develop simultaneously, we should expect to have plants of equal size and vigor.

Discussion of Results

The important question, of course, is this: Do the results have any bearing upon the question of rejuvenescence in plants?

Rejuvenescence in the sense in which Minot (1908), Child (1915), and Conklin (1913) define the term will be used here. They hold that rejuvenescence is a condition of an organism indicated by metabolic activity increased over what it was prior to the change; and that the methods by which such an increased rate may be indicated vary, but the process is always the same—a partial or entire "undifferentiation," or unloading of differentiated products which interfere with maximum metabolism. In a general way it may be said that differentiation and growth are gradual processes, usually having their origin in a single cell. This passage from the simple to the complex in the life cycle of an organism is invariably accompanied by a gradual retardation of the rate of metabolic activity, which finally ceases, and death ensues. Minot aptly says that death is the price paid for differentiation. On the other hand, the process of "undifferentiation" must and undoubtedly does take place. Just what the process is, and how it may be effected, is still largely a matter of conjecture.

It has been quite generally assumed that sexuality is essential to rejuvenation. Child has shown that complex organisms may be partially rejuvenated by a period of partial starvation, and Conklin calls attention to the rejuvenation which apparently takes place in frogs and other animals after a period of hibernation, comparable to the starvation period of Child. He also suggests that such periods of rest and encystment shown by many animals and plants are directly correlated with partial rejuvenation processes. Furthermore, Child has shown that apogamous reproduction is just as effective as gametic reproduction in securing rejuvenescence. Indeed, this would seem to be the case in plants like the banana whose sexual apparatus for unknown periods of time has been inoperative, and yet in which there has been no indication of the plant's "running out."

Now all the evidence gathered in the study of polyembryonic Citrus seedlings seems to indicate that, if we admit that rejuvenescence has occurred at all, it has occurred to the same degree in apogamous and gametic plants. And in the light of the more recent work of Child (1915), Conklin (1913), and Woodruff (1914), we may conclude that sexuality is not necessary to rejuvenation.

That the process of "undifferentiation" and sexuality often occur at approximately the same period in the life cycle of the organism is not denied. Indeed, the reduction or "undifferentiation" of the organism to a single cell is essential before fertilization can occur, but by this reduction rejuvenescence is already effected independent of any fusion which may or may not occur later. Providing the necessary conditions for development surround the single-celled embryo, whether it is stimulated to activity by a sperm, or a needle prick, or certain physiological conditions, is of little moment so far as rejuvenescence is concerned. It is not impossible that the similar physiological conditions surrounding the polyembryonic embryos in their development may explain the similarities noted in the seedlings. The primary function of sexuality it would seem is the production of new character combinations in the offspring through the redistribution of distinctive ones found in the parents. This view is substantiated by the work of Jennings (1912) and Woodruff (1914), who show that protozoa are potentially immortal, and that sexuality serves the sole purpose of producing favorable variations.

SUMMARY

1. Germination tests showed that 43.18 percent of the seeds of *Citrus grandis* produce polyembryonic shoots.
2. A new method for clearing and staining leaves is described.
3. The size of vein-islets is not correlated with the shape and location of the islets in leaves of *C. grandis*.
4. The size of the vein-islets is directly correlated with the maturity of the leaf. From the most immature to the fully matured leaves there is a gradual increase in the size of vein-islets.
5. In mature leaves the size of the vein-islets is quite constant, independent of the size of the leaf.
6. The size of vein-islets in mature leaves from chlorotic, stunted plants is the same as in mature leaves from healthy plants.
7. The size of vein-islets revealed no difference between polyembryonic seedlings arising from a single seed. No doubt the individuals composing some of these polyembryonic pairs were of gametic and apogamous origin respectively.
8. The nucleo-cytoplasmic ratio, as determined in the meristem of the root tips from polyembryonic plants, is essentially the same in gametic and apogamous plants.
9. Difference in size of polyembryonic seedlings seems to be a matter

primarily concerned with priority of development of the embryos, and not directly correlated with sexuality.

10. Rejuvenation seems to be effected equally well through apogamous and through gametic reproduction.

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BIBLIOGRAPHY

Benedict, H. M. 1915. Senile changes in leaves of *Vitus vulpina* L. and certain other plants. N. Y. Agr. Exp. Sta. (Cornell) Mem. **7**: 281–370.

Chamberlain, C. J. 1915. Methods in plant histology. Pp. 1–314. Chicago.

Child, C. M. 1912. The process of reproduction in organisms. Biol. Bull. **23**: 1–39.

——. 1915. Senescence and rejuvenescence. pp. 1–481. Chicago.

Conklin, E. G. 1912. Cell size and nuclear size. Journ. Exp. Zool. **12**: 1–98. fig. 37.

——. 1913. The size of organisms and their constituent parts in relation to longevity, senescence and rejuvenescence. Pop. Sci. Mo. **83**: 178–198.

Coulter, J. M., Barnes, C. R., and Cowles, H. C. 1910. A textbook of botany for colleges and universities. Vol. 1. pp. 1–484. Chicago.

DeVries, H. 1878. Beiträge zur speciellen Physiologie landwirthschaftlicher Culturpflanzen. V. Wachsthumsgeschichte der Kartoffelpflanze. **7**: 591–682. pls. 16–17.

Hertwig, R. 1903. Ueber Korrelation von Zell- und Kerngrösse und ihre Bedeutung für die geschlechtliche Differenzierung und die Teilung der Zelle. Biol. Centralbl. **23**: 194–223.

Jennings, H. S. 1912. Age, death and conjugation in the light of the work on lower organisms. Pop. Sci. Mo. **80**: 563–577.

——. 1913. Effect of conjugation in Paramecium. Journ. Exp. Zool. **14**: 279–391.

Minot, C. S. 1908. The problem of age, growth and death. Pp. 1–280. New York.

Nägeli, C., and Cramer, C. 1855. Pflanzenphysiologische Untersuchungen. I. Heft von Carl Nägeli. **1**: 88–120.

Osawa, I. 1912. Cytological and experimental studies in Citrus. Journ. Imp. Agr. Coll. Tokyo **4**: 83–115. pls. 8–12.

Prantl, K. 1883. Studien über Wachsthum, Verzweigung, und Nervatur der Laubblätter, insbesondere der Dicotylen. Ber. Deutsch. Bot. Ges. **1**: 280–288.

Schuster, W. 1908. Die Blattaderung des Dicotylenblattes und ihre Abhängigkeit von äusseren Einflüssen. Ber. Deutsch. Bot. Ges. **26**: 194–237.

Stevens, W. C. 1907. Plant Anatomy. pp. 1–399. Philadelphia.

Strasburger, E. 1878. Ueber Polyembryonie. Jena. Zeitschr. Naturw. **12**: 647–667. pls. 15–20.

Webber, H. J. 1900. Work of the U. S. Department of Agriculture on plant hybridization. Journ. Roy. Hort. Soc. **24**: 1–18.

Woodruff, L. L. 1914. On so-called conjugating and non-conjugating races of Paramecium. Journ. Exp. Zool. **14**: 575–582.

Zalensky, W. von. 1902. Ueber die Ausbildung der Nervation bei verschiedenen Pflanzen. Ber. Deutsch. Bot. Ges. **20**: 433–440.